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14. ABSTRACT
Pin1 regulates many factors that are relevant to breast cancer, such as c-Jun, c-Myc, cyclin D1, and cyclin E. However the function of Pin1 in a normal cell is still poorly understood. Thus the role of Pin1 in G0/G1 to S-phase progression and genomic instability was examined in this research project.

15. SUBJECT TERMS
Pin1, cell cycle, genomic instability, breast cancer

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INTRODUCTION

The purpose of the training grant was to identify the potential mechanism(s) by which Pin1, a peptidyl-prolyl *cis-trans* isomerase, influences breast cancer progression. In order to accomplish this objective, we sought to identify novel roles for Pin1 in the G0/G1 to S-phase transition of the cell cycle, which is a process invariably deregulated in human breast cancer. Previous research has shown that Pin1 can differentially regulate oncoproteins known to control G1/S, including c-Jun, c-Myc, cyclin D1, and cyclin E [1-4]. However these studies have not addressed why Pin1 is required in mouse embryonic fibroblasts (MEF) for efficient cell cycle re-entry from quiescence upon mitogenic stimulation [5, 6]. Thus we have further characterized how Pin1 influences cell cycle re-entry in primary MEF. This annual summary will provide an up-to-date report on the progress made to examine the role of Pin1 in regulating the MAPK signaling pathway. In addition, we will discuss our ongoing experiments designed to evaluate the role of Pin1 in genomic instability and breast cancer progression.

BODY

Task 2. Evaluate the role of Pin1 in regulating p27 during G0/G1-S progression:

Previously we had reported preliminary findings that suggested Pin1 was a novel regulator of Rb function, i.e. the serum induced phosphorylation of Rb (on S807/S811) was decreased in Pin1$^{-/-}$ MEF. However further experiments demonstrated that the phospho-Rb antibodies (from Cell Signaling, Sigma, Santa Cruz) used for immunoblotting lacked specificity for mouse Rb. These technical difficulties prompted us to continue to investigate potential Pin1 targets upstream of Rb, which could contribute to the cell cycle defects observed in Pin1$^{-/-}$ MEF.

In order to find a molecular mechanism for the cell cycle re-entry defect in MEF null for Pin1, members of the MAPK signaling pathway were analyzed for activation in response to re-stimulation with 10% fetal bovine serum (FBS). The results showed that the Raf/MEK/ERK signaling cascade was fully functional in the absence of Pin1 (Figure 1). In addition, the serum induced expression of c-Fos and c-Jun (two MAPK effectors and Pin1 targets) was identical between Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF (Figure 2). Although there was a minor defect in the induction of c-Myc, which has been published [3], the general lack of detectable differences between WT and Pin1 KO MEF was perhaps unsurprising given the mild cell cycle phenotype obtained when using 10% FBS as the mitogenic stimulus (Figure 3A). We speculated that the presence of many undefined growth factors in FBS could mask the effects of Pin1 deletion via crosstalk.

To avoid issues of crosstalk and evaluate the role of Pin1 in modulating a specific growth signaling pathway, we chose fibroblast growth factor (FGF) to stimulate serum starved MEF. Although the cell cycle phenotype was still mild (Figure 3B), the FGF induced phosphorylation of p90^{RSK} was reduced in the absence of Pin1 (Figure 4). This defect occurred despite normal ERK activation, which suggested that Pin1 may influence ERK signaling through p90^{RSK}. This work is currently ongoing in collaboration with Theresa Barberi, who is investigating a protein synthesis defect in Pin1$^{-/-}$ MEF (Figure 5). These studies will provide novel insights into how Pin1 is involved in G0/G1-S progression, which appears to include the control of protein synthesis.

Task 3. Examine the role of Pin1 in human breast cancer:

In order to investigate the role of Pin1 in human breast cancer, we are still currently developing two human cell lines using HMEC and WI38 cells, which will be used in soft agar assays and *in vivo* tumorigenesis models. These lines will stably overexpress Pin1 mRNA or Pin1 shRNA along with differing combinations of oncogenes (hTERT, p53^{DD}, cyclin D1, cdk4^{R24C}, c-Myc^{T58A} and/or H-Ras^{G12V}) known to be sufficient to transform human cells [7, 8]. This new six gene model of transformation is particularly useful for this study, since p53, cyclin D1 and c-Myc are all Pin1 targets. These studies will clarify some of the controversy over Pin1's role in cancer, i.e. a tumor promoter or a tumor suppressor, reviewed in [9]. We hypothesize that Pin1 will have differential roles in cellular transformation depending on genetic background and tissue specific differences.

To further test this hypothesis, we are also developing MEF cell lines derived from C57BL/6-J, 129Sv and B6129F1 genetic backgrounds that stably express Pin1 shRNA. After transduction with p53^{DD} and H-Ras^{G12V}, these lines will be assayed for the development of aneuploidy and cellular transformation. Our most recent data demonstrated that Pin1 shRNA can accelerate the development of aneuploidy induced by p53^{DD} expression in MEF from the C57BL/6-J strain (Figure 6), which is consistent with previous studies using Pin1$^{-/-}$ MEF [4]. These ongoing studies will ascertain if Pin1 could be a useful therapeutic target or a diagnostic marker for tailored therapeutic strategies to treat breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of Pin1 as a novel regulator of p90^{RSK}.
- Development of novel reagents to evaluate the role of Pin1 in cancer.

REPORTABLE OUTCOMES

Poster Abstracts:

Lew, B.O., T. Barberi and A.R. Means (Sept. 2008). Pin1 influences the timing of the G0/G1 to S-phase transition in mouse embryonic fibroblasts. Duke University Medical Center, Department of Pharmacology and Cancer Biology Annual Retreat, NC. (see Appendices)

CONCLUSION

Although these studies are still preliminary, the data suggest that Pin1 is involved in regulating protein synthesis through the regulation of p90^{RSK}. Protein synthesis is required for the transition from G0/G1 into S-Phase, and is also a process important for tumorigenesis. Hence it appears that inhibition of Pin1 should attenuate both processes and perhaps be a novel therapeutic target. On the other hand, it seems that in certain genetic contexts Pin1 may function as a tumor suppressor and be involved in maintaining genomic integrity. Therefore much more work is required to elucidate the role of Pin1 in human breast cancer.

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APPENDICES

Poster Abstracts:

Pin1 influences the timing of the G0/G1 to S-phase transition in mouse embryonic fibroblasts.

Brian O. Lew, Theresa Barberi and Anthony R. Means
Department of Pharmacology and Cancer Biology, Duke University

Pin1 is an evolutionarily conserved peptidyl-prolyl isomerase that specifically recognizes and catalyzes the *cis-trans* isomerization of phospho-Ser/Thr-Pro bonds. Pin1 has been shown to regulate the stability, localization and function of several different proteins, including c-Jun, c-Myc, cyclin D1 and cyclin E. These Pin1 targets are known regulators of cell cycle progression, and highlight the potential importance of Pin1 in the G0/G1 to S-phase transition. Indeed, Pin1 $^{-/-}$ mouse embryonic fibroblasts (MEF) exhibit an impaired ability to transit from quiescence (G0) into S-phase. However, although Pin1 $^{-/-}$ MEF show a one hour delay in cell cycle re-entry, their re-entry kinetics were similar to wildtype counter parts. So perhaps not surprisingly, our recent data suggest that Pin1 is not involved in the regulation of the MAPK pathway or DNA replication during S-phase, which implied a role for Pin1 in G1 progression prior to S-phase entry. Preliminary analysis of the Rb pathway, which controls G1 to S-phase progression, revealed impaired cyclin D1 expression and phosphorylation of Rb on S780. Therefore Pin1 might influence G1 progression via direct or indirect regulation of the Rb pathway.

SUPPORTING DATA

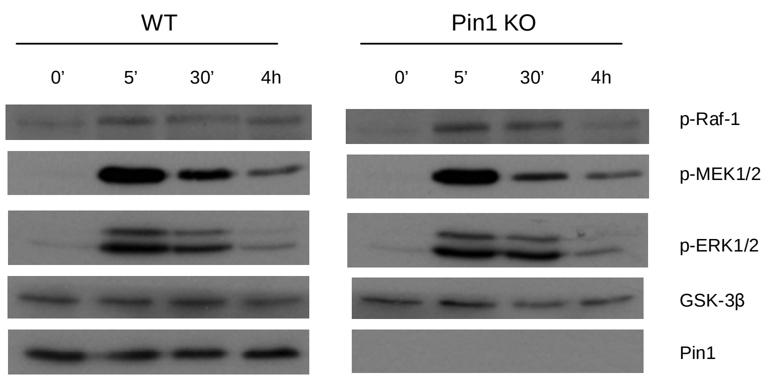

Figure 1. Loss of Pin1 does not affect activation of the Raf/MEK/ERK signaling cascade. Asynchronously growing Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF were serum starved for 24h. The cells were re-stimulated with 10% FBS and harvested at the indicated times for western blot analysis. GSK-3β served as a loading control.

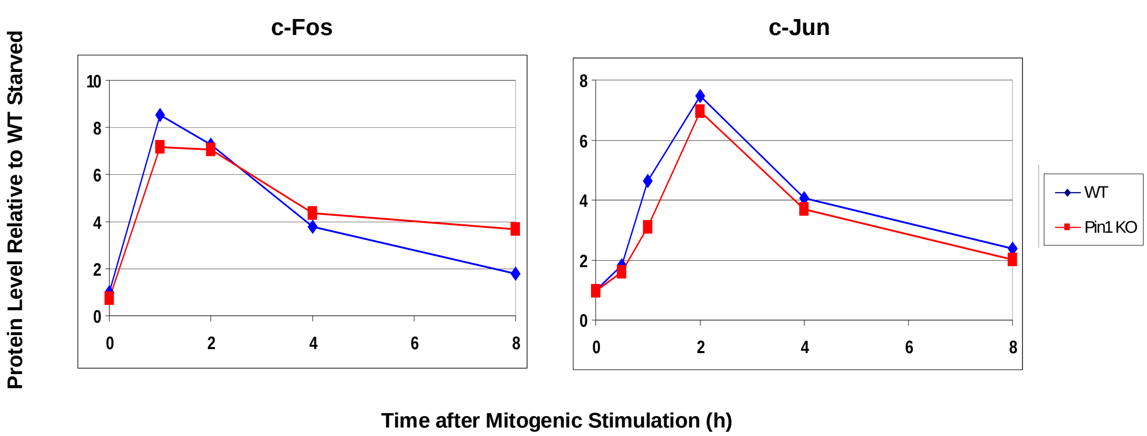

Figure 2. Loss of Pin1 does not impair serum induced expression of c-Fos or c-Jun. Asynchronously growing Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF were serum starved for 24h. The cells were re-stimulated with 10% FBS and harvested at the indicated times for western blot analysis using Licor technology. Actin served as a loading control. Normalized values were plotted in the graphs.

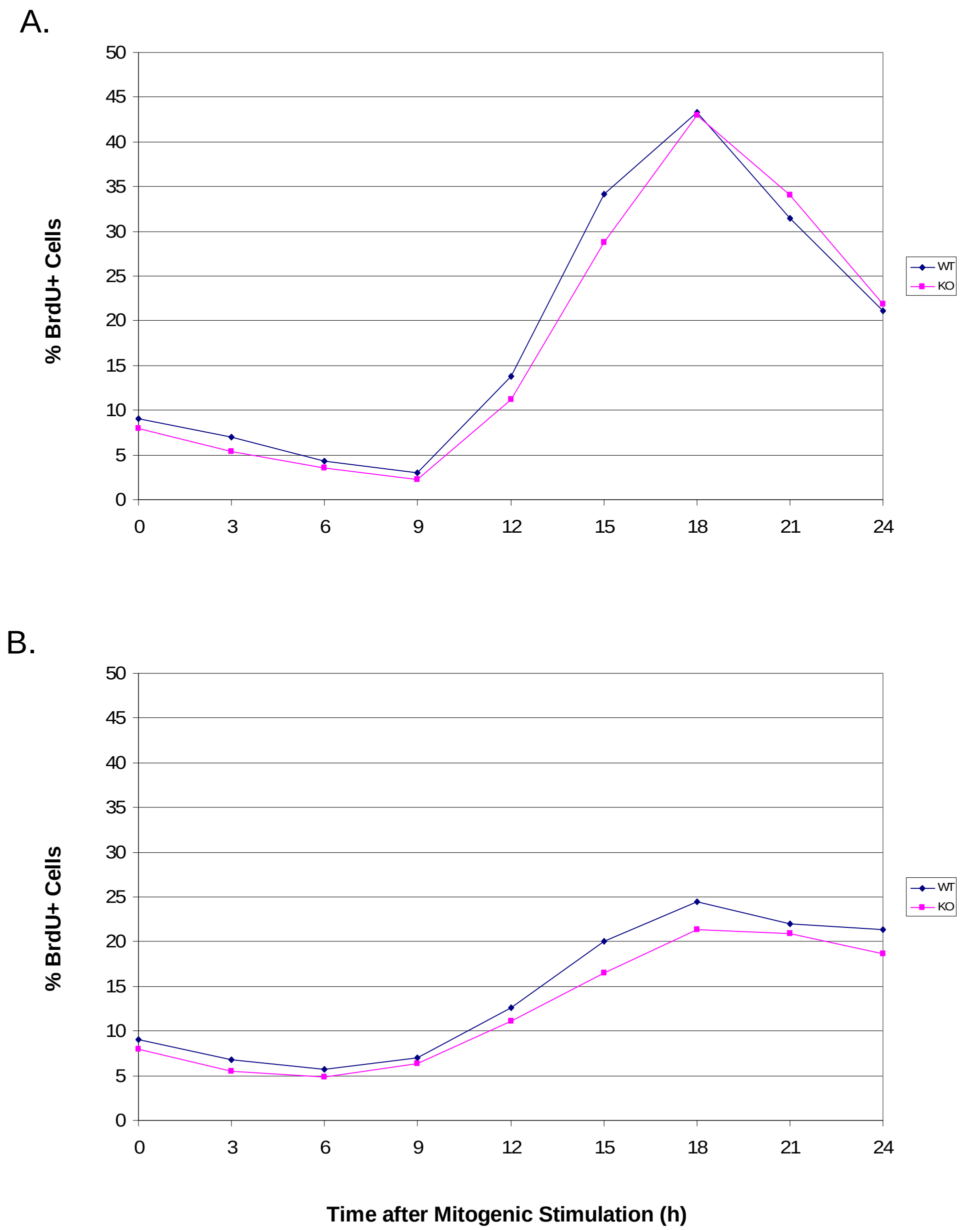

Figure 3. Loss of Pin1 leads to impaired cell cycle re-entry in primary MEF. Asynchronously growing $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF were serum starved for 24-26h. The cells were re-stimulated with either (A) 10% FBS or (B) 2.5 ng/ml FGF, then pulse labeled with BrdU and harvested at the indicated times. Samples were analyzed for BrdU incorporation by flow cytometry.

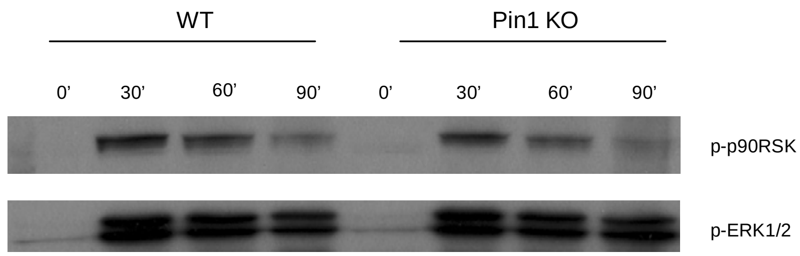

Figure 4. Loss of Pin1 impairs activation of p90RSK, but not ERK1/2. Asynchronously growing Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF were serum starved for 24h. The cells were re-stimulated with 2.5 ng/ml FGF and harvested at the indicated times for western blot analysis.

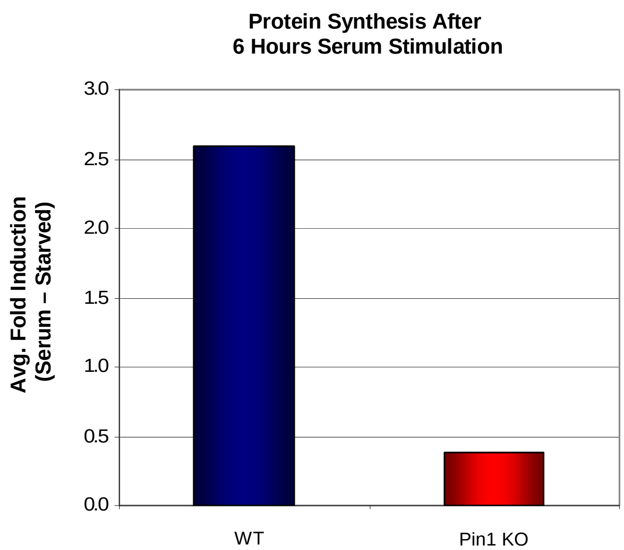

Figure 5. Loss of Pin1 impairs protein synthesis. MEF were serum starved for 48h, switched to methionine-free media for 30 min, and then supplemented with 35S-methionine in the presence or absence of 10% serum for the indicated times. TCA precipitation was performed on 10ug total protein and CPM were calculated using a scintillation counter. Starved (basal) CPM were then subtracted from serum stimulated CPM at each time point.

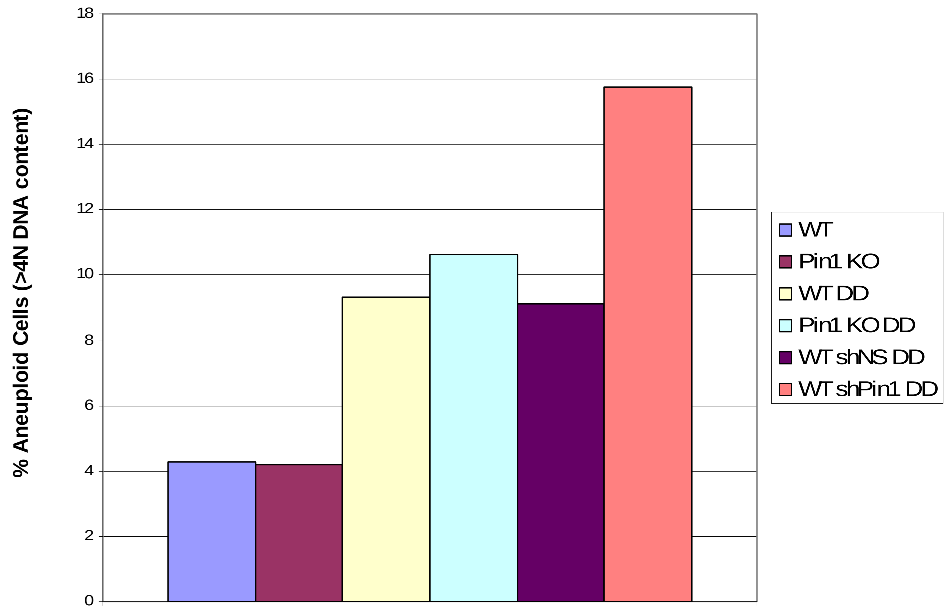

Figure 6. Loss of Pin1 accelerates the development of aneuploidy induced by p53DD expression in MEF. Asynchronously growing MEF were harvested and fixed in cold 70% ethanol. Cells were stained with PI and analyzed by flow cytometry. shNS = non-silencing control.